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Influence of Plant Severing on Movement of *Ostrinia nubilalis* Larvae in *Zea mays* Hybrid Seed Production Fields

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ABSTRACT Genetically engineered corn hybrids that contain a *cry* gene from the bacterium *Bacillus thuringiensis* Berliner (Bt) are gaining popularity for controlling the corn pest *Ostrinia nubilalis* (Hübner). Continuous use of Bt corn, however, could select for *O. nubilalis* that are resistant to this corn. Monitoring for insect resistance is important, because it could help maintain the Bt technology. A possible monitoring method is to collect larval insects in commercial drying bins after harvest from Bt seed production fields. A drawback to this method is that these collections may be contaminated by insects that moved as later instars from severed non-Bt male rows into the adjacent Bt female rows. These larvae have little to no exposure to Bt toxin, resulting in possible "false positives." The objectives of this study were to first find which combination of planting and severing dates produces the least number of larvae that move from non-Bt male plants to Bt female plants and to assess *O. nubilalis* larval movement from severed non-Bt male rows to Bt female rows. Field studies in 2002 and 2003 were designed to simulate a hybrid seed production field. Results suggest that movement of *O. nubilalis* larvae from male corn is minimized when corn is planted early and male plants are severed by 2 wk post-anthesis. This reduces the likelihood of false positives by reducing the number of susceptible larvae moving between Bt and non-Bt plants. Also, larvae moved to all four female rows that were adjacent to the severed rows, but there were significantly more larvae found in the closest row compared with the other three. These results could be used to develop a monitoring program to find *O. nubilalis* larvae with resistance to Bt corn in field populations of *O. nubilalis*.

KEY WORDS Bt, *Bacillus thuringiensis*, European corn borer, resistance management, monitoring

The European corn borer, *Ostrinia nubilalis* (Hübner), has been a serious economic pest of corn, *Zea mays* L., in the United States since its introduction in the early 1900s (Hodgson 1928). Many methods have been used to control *O. nubilalis*, including cultural practices; host plant resistance; rescue treatments with chemical and biological insecticides, particularly the bacterium *Bacillus thuringiensis* Berliner (Bt) (Mason et al. 1996); and recently genetically engineered corn, which expresses a *cry* gene from *B. thuringiensis* (Gordon-Kamm et al. 1990). These plants produce a Cry protein that kills *O. nubilalis* larvae and reduces the need for other types of control. Since commercial introduction in 1996, popularity of Bt corn hybrids has increased; during 2006, 40% of field maize in the United States was planted in Bt varieties (USDA–NASS 2006). With a combination of expanding acreage and season-long expression of Cry proteins there is a need to manage *O. nubilalis* resistance to Bt corn. If the Bt technology is overused, insects could evolve resistance, and Bt corn could become ineffective.

To keep field populations of *O. nubilalis* from developing resistance to lepidopteran-active Bt corn, the Environmental Protection Agency (EPA) requires a refuge of 20% non-Bt crop within 0.8 km of Bt cornfields (EPA 2001). A refuge produces *O. nubilalis* that are susceptible to Bt corn. Theoretically, a sufficient number of genetically susceptible moths will mate with rare resistant moths from the Bt corn and reduce the chance that resistant moths will mate with each other (Tabashnik and Croft 1982). Monitoring for resistant insects is needed to assess whether insect resistance management (IRM) strategies are effective. Methods proposed to monitor for *O. nubilalis* resistance are diagnostic dose (Marçon et al. 2000), F_2 screen (Andow and Alstad 1998), and infield screen (Venette et al. 2000). These methods could be improved if they were cheaper, less labor-intensive, and quicker to process (Caprio et al. 2000). Another problem encountered in monitoring programs is difficulty in adequately sampling a large area for resistant individuals (Bolin et al. 1998).

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A proposed method of resistance monitoring is the bin-monitoring method (Prasifka et al. 2006). This method takes advantage of the existing system used in hybrid seed production where whole ears are harvested and dried (Jugenheimer 1976). Seed production fields are planted with strips (usually four rows) of female plants of an inbred line, then strips of male plants from an inbred line. This alternating pattern of male and female strips is repeated over an entire field. After pollination, the male rows are destroyed (chopped or mowed, but usually left in the field), but the timing of this destruction varies. Ears of the female plants are harvested and transported to drying bins where they are dried. Some of the *O. nubilalis* larvae that are commonly found in the ears will move out during the drying process and fall below a drying partition to the bin floor. *O. nubilalis* larvae found in female Bt ears during harvest are potentially resistant. These larvae are collected, brought into the laboratory, reared to adults, and mated with susceptible laboratory colony to produce the F_1 generation. Similar to the in-field screen method, an inbred F_2 generation is produced by sib-mating F_1 individuals. These larvae then undergo a diagnostic-dose assay.

There is the possibility, however, that these insects could have developed on non-Bt corn from the male rows, and then they moved into the Bt female rows. In this case, if the *O. nubilalis* larvae that move from non-Bt plants to Bt plants were late instars, they could survive (Walker et al. 2000). Such larvae would decrease the efficiency of the bin-monitoring method, because they would incorrectly be considered candidates for Bt resistance (i.e., false positives). If false positives could be reduced or eliminated to acceptable levels, then collecting *O. nubilalis* larvae in this manner could develop into an inexpensive and labor-efficient method of monitoring for *O. nubilalis* resistance to Bt corn. The objectives of this study were to determine the best combination of planting and severing dates to reduce movement of *O. nubilalis* from male to female rows in corn hybrid production fields and to assess *O. nubilalis* larval movement from severed non-Bt male rows to Bt female rows.

Materials and Methods

Corn Varieties. Field experiments were conducted in summers 2002 and 2003 near Ames, IA, at Iowa State University's Johnson and Marsden farms, respectively. Field experiments were conducted using a hybrid seed production field design. Seed was planted with a four-row planter. The field corn variety for female and border rows was Pioneer 36R11, a 102-d Bt hybrid (Mon810 event, Pioneer HI-Bred International, Johnston, IA). The popcorn hybrid used for male rows was McHone's variety M2101, a non-Bt hybrid with a 102-d maturity (McHone Seed Co., Ames, IA). Popcorn was selected because it is highly attractive to *O. nubilalis* for oviposition resulting in high numbers of larvae per plant, thus increasing the potential for larval movement.

Experimental Design. Experiments consisted of nine treatments (2002) or six treatments (2003) that were each organized in a split-plot design, with planting date as main plot and severed dates as subplots. Planting dates were randomized, and the severed dates were randomly assigned for each planting date (Fig. 1). There were eight replicates. Plots were similar to the configuration of a hybrid production field, by starting with two rows of male corn (popcorn). Then, on both sides of the popcorn there were four rows of female corn (Bt corn). Finally, on both sides of the female rows there were four border rows of Bt corn. Border rows were used to reduce edge effects and to isolate plots. In 2002, row lengths were 34.1 m with sections of treatments that were 4.9 m. Row lengths were 45.7 with 7.6-m sections of treatment in 2003. Treatment sections had 4.9- and 7.6-m buffers in 2002 and 2003, respectively, to reduce the possibility of treatment interactions.

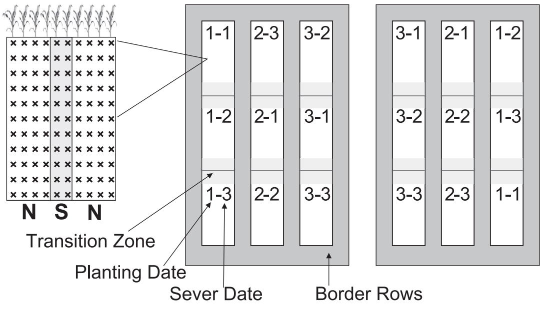

Fig. 1. Experimental design showing two of the eight blocks, and nine planting and sever date combination treatments for 2002; transition zone between plots and border rows around plots are indicated. Inset shows two rows of severed corn (S) with two four row strips of corn not severed (N).

There were three planting dates in 2002 (8 May, 30 May, and 17 June) and two planting dates in 2003 (19 May and 5 June). Within each of the planting dates, there were three randomly assigned sever dates. The severed dates were 1, 2, and 4 wk after the end of male plant anthesis. Corn plants were monitored daily once the corn had reached the VT (tasseling) stage to determine the end of anthesis (Ritchie et al. 1997).

During the last week of June and the first week of July, possible off-type plants in the Bt corn were eliminated by infesting ≈25 larvae into the whorl during the V6 stage (Ritchie et al. 1997) of each plant by using the inoculator method (Davis and Oswalt 1979). Plants were examined ≈1 wk after infestation. To error on the side of caution, any plants that had damage resembling shot holes were designated off-types, and they were removed from the plot. After this initial infestation, all *O. nubilalis* infestations that occurred were natural.

On each of the sever dates, rows of designated male corn had a 4.9-m (2002) or 7.6-m (2003) section severed. Male plants were severed with lopping shears, the plants were placed on the ground in the rows from which they were severed, and care was taken so that severed plants did not touch plants in the female rows. After male row severing, five randomly selected plants

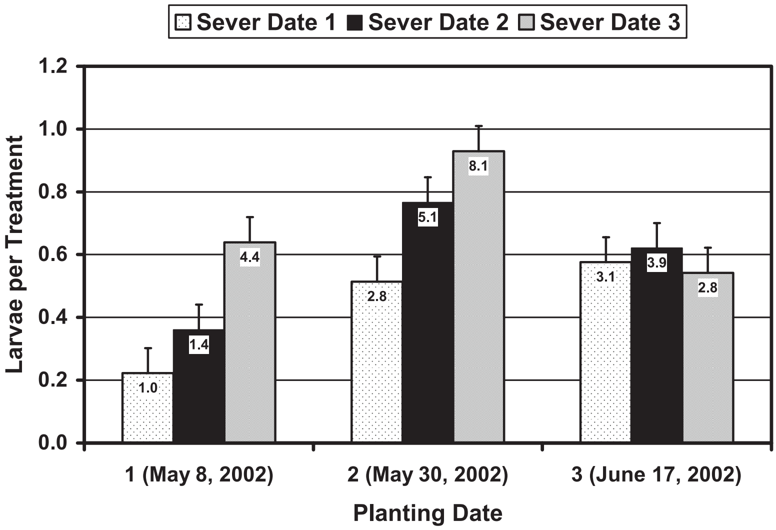

Fig. 2. Transformed mean total number (±SE) of *O. nubilalis* larvae (log 10 + 1) at each planting and sever date combination for 2002. The numbers at the top of each bar represent the untransformed mean number of larvae per treatment.

were destructively sampled by splitting stalks. Number of *O. nubilalis* larvae in each plant and presence of damage were recorded. Destructive sampling continued on the severed male plant rows, sampling five plants a week until harvest.

Starting in late August, the female rows adjacent to the severed male rows in each plot were sampled. Samples were taken on the four rows (distances were row 1, 0.8 m; row 2, 1.5 m; row 3, 2.3 m; and row 4, 3.1 m) of corn on both sides of the severed male corn. Number of *O. nubilalis* larvae and damage associated with larvae were recorded. All plants in the first row were destructively sampled. Five random plants were destructively sampled in each of second, third, and fourth rows. Shanks and ears of plants in each row that were not destructively sampled were examined for larvae and presence of damage.

Data Analysis. The cumulative number of larvae collected within each plot (n) for sever date and planting date combinations were transformed using log 10 ($n + 1$) to meet assumptions of normality and homoscedasticity of variances for analysis of variance (ANOVA).

Two-way ANOVA was used to assess the effect of planting date and sever date on number of larvae found in severed male rows in the split-plot design. The main plot was planting date and the subplots were sever dates. Restricted maximum likelihood (REML) estimates of sources of variances in the mixed model were estimated with PROC MIXED, SAS version 8.2 (Littell et al. 1996). The dependent variable was the total number of larvae, and the fixed effects were planting date, sever date, and their interaction. Block effects were considered random. Least-squares means for main effects were separated using LSMEANS statement of PROC MIXED and protected least significant difference (LSD) (presented as t values, $P < 0.05$). To reduce the experiment-wise type I error, only a subset of treatment comparisons were evaluated for significant planting date × sever date interactions. The slice option of the LSMEANS statement was used to test for overall differences among severed date treatments within each planting date (SAS Institute 1999).

An REML–ANOVA model was examined (Littell et al. 1996) to assess the number of larvae found in the four female rows from severed male rows. The dependent variable was mean number of larvae per plant; planting date, sever date, row, and all interactions were fixed effects. Block was designated random. Treatment means were separated using LSMEANS option. Contrast statements also were used to compare the mean number of larvae in row 1 to the other three rows and differences among rows 2, 3, and 4.

Results

Sever Date Study. *Field Research 2002.* Cumulative numbers of larvae were significantly affected by planting dates ($F = 8.59$; df = 2, 14; $P = 0.0037$), sever dates ($F = 11.2$; df = 2, 42; $P < 0.0001$), and the interaction between planting date and sever date ($F = 3.91$; df = 4, 42; $P = 0.0086$) (Fig. 2). Sever date 1 plots contained significantly fewer cumulative number of larvae than plots from sever dates 2 ($t = 2.58$, df = 42, $P = 0.0134$) and 3 ($t = 4.73$, df = 42, $P < 0.0001$). Sever date 2 plots had significantly fewer larvae than sever date 3 plots ($t = 2.15$, df = 42, $P = 0.038$). Fewer cumulative number of larvae were found from planting date 1 plots compared with those from planting date 2 ($t = 4.14$, df = 14, $P = 0.0010$) and planting 3 ($t = 2.16$; df = 14; $P = 0.048$). There were no significant differences found between planting dates 2 and 3 ($t = 1.98$, df = 14, $P = 0.068$).

Significant planting date × sever date interactions were due to differences among sever dates for planting dates 1 ($F = 9.48$; df = 2, 42; $P = 0.0004$) and 2 ($F =$

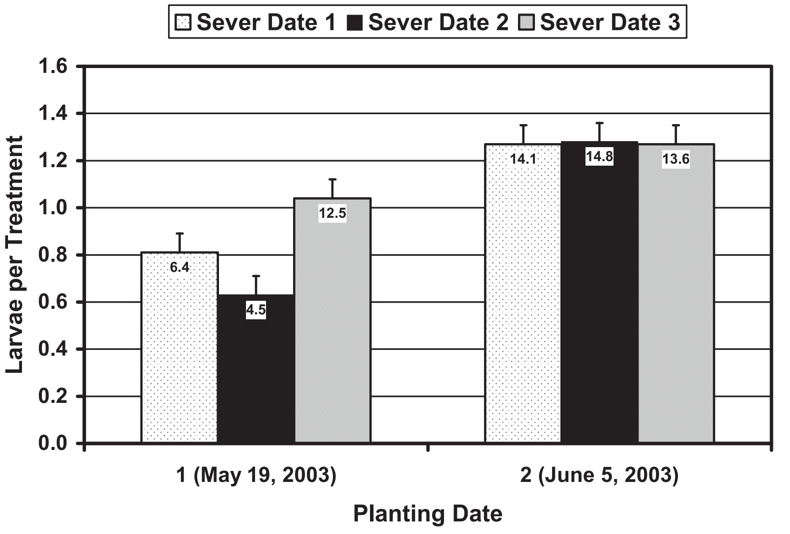

Fig. 3. Transformed mean total number (±SE) of *O. nubilalis* larvae (log 10 + 1) at each planting and sever date combination for 2003. The numbers at the top of each bar represent the untransformed mean number of larvae per treatment.

9.23; df = 2, 42; $P = 0.0005$), whereas there were no significant differences among sever dates in planting date 3 ($F = 0.33$; df = 2, 42; $P = 0.72$). The combination of planting date and sever date that contained the least mean number of larvae was planting date 1 with sever date 1 (Fig. 2). Planting date 2 and sever date 3 plots contained the greatest mean number of larvae (Fig. 2).

Field Research 2003. Cumulative numbers of larvae were significantly affected by planting dates ($F = 39.4$; df = 1, 35; $P < 0.0001$), sever dates ($F = 5.89$; df = 2, 35; $P = 0.0062$), and the interaction between planting date and sever date ($F = 7.63$; df = 2, 35; $P = 0.0018$) (Fig. 3). Differences of least-squares means show that there were significantly more larvae in sever date 3 plots than either of the sever date 1 ($t = 2.32$; df = 35; $P = 0.027$) or 2 plots ($t = 3.35$; df = 35; $P = 0.0019$). There were no significant differences between sever dates 1 and 2 ($t = 1.04$, df = 35, $P = 0.31$). There were significantly fewer larvae in plots planted 19 May compared with plots planted 5 June ($t = 6.28$, df = 35, $P < 0.0001$).

Planting date × sever date interactions were caused by significant differences among sever dates for planting date 1 ($F = 13.5$; df = 2, 35; $P < 0.0001$) and no significant differences among sever dates in planting date 2 ($F = 0.07$; df = 2, 35; $P = 0.94$). The combination of planting date and sever date that had the least cumulative number of larvae was planting date 1 with sever date 2 (Fig. 3).

Larval Movement Analysis. *Field Research 2002.* Mean number of larvae moving out of severed rows into female

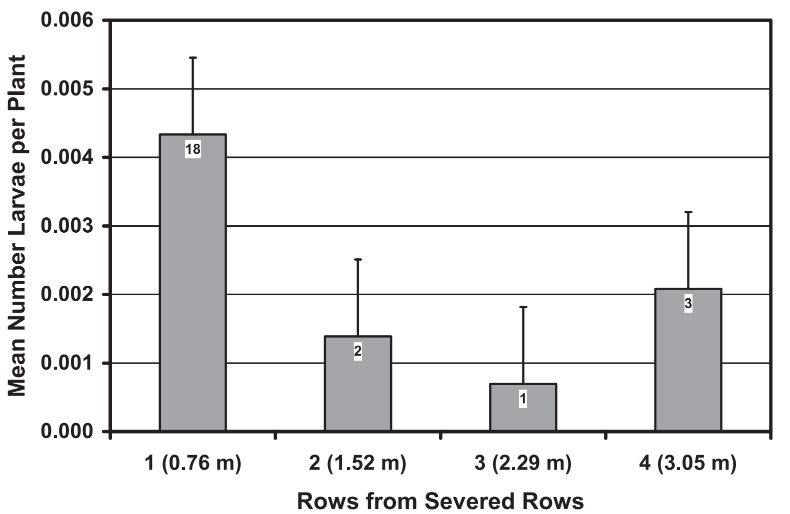

Fig. 4. Mean number (±SE) of *O. nubilalis* larvae found in 2002 at four distances from the severed male rows. The numbers at the top of each bar represent the total number of larvae found in each row over all blocks.

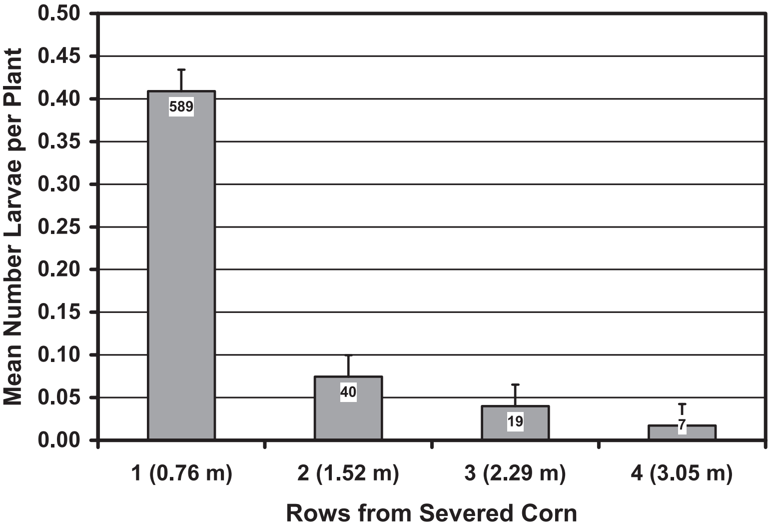

Fig. 5. Mean number (±SE) of *O. nubilalis* larvae found in 2003 at four distances from the severed male rows. The numbers at the top of each bar represent the total number of larvae found in each row over all blocks.

rows was significantly affected by sever date ($F = 3.24$; df = 2, 231; $P = 0.041$). There were no significant differences found in planting date ($F = 1.08$; df = 2, 21; $P = 0.3569$) or in row number ($F = 2.07$; df = 3, 231; $P = 0.10$). Two-way and three-way interactions also were not significant. Least-square-mean estimates of larvae per plant for each row are found in Fig. 4.

The mean number of larvae decreased greatly from the first row to the other three rows. Row 1 had significantly more larvae compared with rows 2, 3, and 4 ($F = 5.41$; df = 1, 231; $P = 0.02$), but rows 2, 3, and 4 were not significantly different from each other ($F = 0.40$; df = 2, 231; $P = 0.67$). The total number of larvae during 2002 was very low due to low *O. nubilalis* populations.

Field Research 2003. Mean number of larvae moving out of severed rows into female rows was significantly affected by row number ($F = 74.3$; df = 3, 134; $P < 0.0001$), and the three-way interaction (planting date × sever date × row number; $F = 2.75$; df = 6, 134; $P = 0.015$). There were no significant differences in planting date ($F = 0.08$; df = 1, 5.94; $P = 0.79$), sever date ($F = 0.69$; df = 2, 141; $P = 0.51$), or in the two-way interactions. Least-squares mean estimates of larvae per plant for each row are found in Fig. 5.

The mean number of larvae decreases greatly from the first row to the other three rows. Row 1 had significantly more larvae compared with rows 2, 3, and 4 ($F = 219.3$; df = 1, 134; $P < 0.0001$), but rows 2, 3, and 4 were not significantly different from each other ($F = 1.84$; df = 2, 134; $P = 0.16$).

Contrast statements also were used to partition the three-way interaction of planting date × sever date × row number into testable hypotheses. The relative differences in the mean number of larvae in row 1 versus rows 2 through 4 varied among planting date and sever date treatments (planting date × sever date × (row 1 versus rows 2, 3, and 4; $F = 7.16$; df = 2, 134; $P = 0.001$). Relative changes in the mean number of larvae in rows 2, 3, and 4 were similar among planting date and sever date treatments (planting

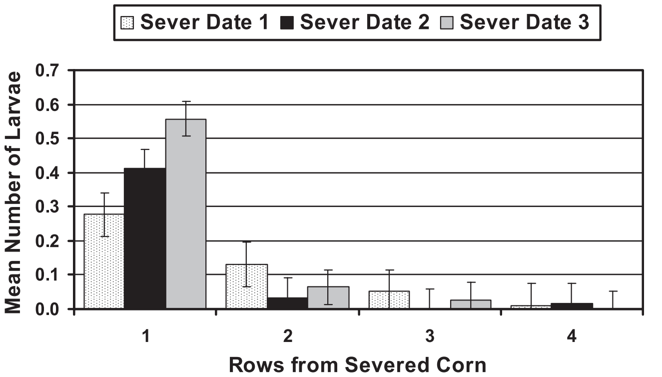

Fig. 6. Mean number of larvae per plant found at each distance from male rows separated by each sever date for planting date 1 in 2003.

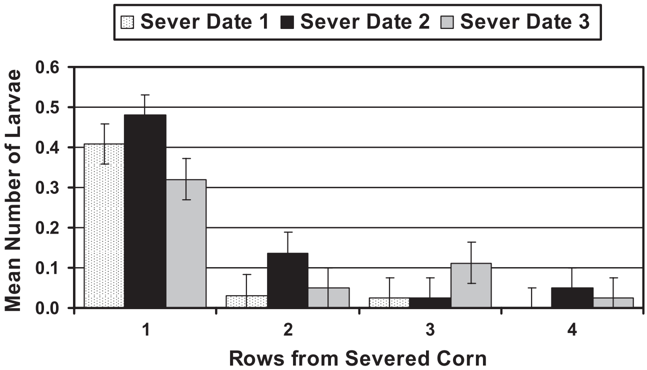

Fig. 7. Mean number of larvae per plant found at each distance from male rows separated by each severed date for planting date 2 in 2003.

date × sever date × remaining row number interactions; $F = 0.54$; df = 4, 134; $P = 0.70$). The interaction seems to be due to planting date 1 and sever date 1. Figures 6 (planting date 1) and 7 (planting date 2) show that going from row 1 to row 4 there is a large dropoff in the number of larvae for all sever dates, but there is a more gradual decline of the number of larvae for planting date 1 and sever date 1.

Off-Type Tests. In testing for Bt off-types in 2002, there were 88 corn plants designated off-types of ≈60,000 corn plants. In 2003, there were 25 corn plants of ≈90,000 corn plants designated off-type.

Discussion

Monitoring *O. nubilalis* for resistance to Bt proteins becomes all the more important as use of Bt corn increases. Monitoring for *O. nubilalis* resistance is focused on areas in the Corn Belt with high use of Bt corn; currently these include three areas: northern Illinois to central Iowa; northwestern Iowa, northeastern Nebraska, eastern South Dakota, and southwestern Minnesota; and southwestern Kansas, panhandle of Oklahoma and northern Texas (Matten et al. 2004). Increasing the number of locations would be easier if more efficient monitoring methods, such as bin monitoring, were developed. This study addresses an important question that needs to be answered to make the bin-monitoring system plausible: What combination of planting date and sever date minimizes larval movement from male plants to female plants.

Larval movement, particularly late instars, from non-Bt plants to Bt plants would compromise the efficiency of the bin-monitoring method because such larvae would increase the number of false positives. In the current study, larval movement was minimized by planting the corn early and severing the male plants within 2 wk postanthesis. Fewer larvae occurred in these plants because second generation *O. nubilalis* females are not as likely to oviposit in early planted corn because the corn had matured past the preferred phenological stage (Mason et al. 1996). Destroying male corn early after anthesis prevents the plants from being available for oviposition, as well as reducing the food quality for any developing *O. nubilalis* larvae. Also, findings in this study indicated that when planting occurred after May, the number of larvae was high for all severing dates, which suggests second generation *O. nubilalis* females were highly attracted to these plants. This finding also suggests the larvae were able to continue developing on corn plants even after the plants were severed, and, in some cases, after plants had advanced stages of decomposition. Larvae moved into all four female corn rows, but there were significantly more larvae in the first row versus rows 2, 3, and 4. This confirms observations of Ross and Ostlie (1990), that most larvae move only one row (0.8 m) from their original plant.

One problem with the bin-monitoring method is that conditions in drying bins are harsh for *O. nubilalis* larvae due to the high temperatures and dry air. Survival is low, and those larvae that do survive bin drying have low survival through diapause (Prasifka et al. 2006). Thus, methods are needed to increase the survival of these larvae, so they can be reared to adults. After considering Prasifka et al. (2006) and this study, perhaps a combination of bin-monitoring and in-field-screen methods could be developed. Such a monitoring method would involve sentinel fields planted with Bt corn attractive to *O. nubilalis*, along with a control, non-Bt near-isoline corn, that would be planted separately, but nearby. Preferably the Bt trait would be linked to a herbicide tolerance trait so that off-type plants could be removed with the appropriate herbicide. Corn ears could be harvested from sentinel fields and moved into drying bins. The ears then could be dried under favorable conditions for larvae, thus improving survival. Larvae from the Bt corn would be brought into the laboratory and subsequent diagnostic or F_2 tests would be conducted on their progeny. Larvae from the non-Bt corn would be used to assess *O. nubilalis* populations.

These studies fit into a larger integrated pest management project based on the Bt Maize Economic Tool or BET program, which uses models based on both insect and corn phenology (Hellmich et al. 2005). The models designed by D. D. Calvin, J. Hyde (The Pennsylvania State University, University Park, PA), and J. M. Russo (ZedX, Inc., Bellefonte, PA) determine the best time to plant corn to either maximize or minimize its attractiveness for any flight of *O. nubilalis* adults. In hybrid production fields, the BET models could be used to determine the best planting dates in an area to help minimize larval movement among Bt and non-Bt plants. In sentinel Bt fields, the models could be used to determine the best corn maturities and planting dates to maximize the number of *O. nubilalis* larvae present for monitoring purposes.

Monitoring *O. nubilalis* resistance to Bt corn increasingly will be more challenging as new types of Bt corn are introduced into the market. Bin monitoring or the combination in-field-screen bin-monitoring method, especially used with the BET models, could be useful tools to help meet this challenge.

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